EUCALYPT KRAFT PULP DELIGNIFICATION WITH POLYOXOMETALATE ASSISTED BY FUNGAL VERSATILE PEROXIDASE

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Abstract

Polyoxometalates have been reported as alternative redox catalysts for pulp delignification in environmentally-friendly bleaching sequences. Enzymatic catalysis is a promising approach to regenerate polyoxometalates that are highly effective in delignification, but very difficult to be subsequently reoxidized by O_2 and other oxidants. In this work, the oxidation of a manganese-substituted polyoxometalate (POM) $[SiW_{11}Mn^{II}(H_2O)O_{39}]^{6-}$ ($SiW_{11}Mn^{II}$) by Mn-oxidizing versatile peroxidase (VP) to give the $[SiW_{11}Mn^{III}(H_2O)O_{39}]^{5-}$ ($SiW_{11}Mn^{III}$) species, being active in selective kraft pulp delignification, was studied. The $SiW_{11}Mn^{II}$ species, formed upon lignin oxidation, was quickly oxidized by VP even at room temperature in the presence of H_2O_2 (K_m= 6.4±0.7 mM and k_{cat}= 47±2 s^{-1}). This allowed 95-100% reoxidation of reduced POM with VP/H_2O_2 in the filtrate from eucalypt pulp delignification. In this way, it was possible to reuse the liquor from a $SiW_{11}Mn^{III}/O_2$ stage for further delignification, in a sequence constituted by two POM stages, with a short intermediate step consisting of the addition of VP/H_2O_2 to the filtrate for $SiW_{11}Mn^{II}$ reoxidation. When the first D stage of a conventional DEDED bleaching sequence was substituted by the two-stage delignification with POM assisted by VP (POM-VP-POM), a 50% saving in ClO_2 consumption was obtained for similar mechanical strength of the final pulp (89% ISO brightness).

1. Introduction

Polyoxometalates (POMs) are recognised regenerable agents/catalysts for the higly selective and environmentally sound delignification of kraft pulps (Weinstock et al. 1997; Gaspar et al. 2007). When POMs are used as catalysts, they oxidise residual lignin in pulp and are re-oxidised in turn by oxygen or other oxidizing agent in the same process step. Among POMs applied for the pulp delignification catalysis, $[SiW_{11}Mn^{III}(H_2O)O_{39}]^{5-}$ ($SiW_{11}Mn^{III}$) is known as robust "inorganic porphyrin" and selective reagent/catalyst in the aerobic oxidation of residual lignin in kraft pulp (Weinstock et al. 1997; Gaspar et al. 2003). However, after the lignin oxidation, reduced POM $[SiW_{11}Mn^{II}(H_2O)O_{39}]^{6-}$ ($SiW_{11}Mn^{II}$) is practically impossible to reoxidize neither by O_2, H_2O_2 or O_3 even at high temperatures, which impede its practical application. One of the solutions to break the thermodynamic barrier in POMs re-oxidation with oxygen was found via biocatalysis with laccase, a ligninolytic enzyme of the multicopper oxidase family (Gamelas et al. 2005). In the POM-laccase (POM/L) system, like with known organic mediators, POM oxidises the residual lignin in pulp and reduced POM is re-oxidised with laccase at the same process step. Unlike organic mediators, POM is stable over the pulp treatment in the presence of laccase and can be re-used (Gamelas et al. 2005). Although laccase easily oxidized V^{IV} to V^{V} in vanadium-substituted POMs, the oxidation of Mn^{II} to Mn^{III} in $SiW_{11}Mn^{II}$ was rather slow, with less than 50% reoxidation after 4 h at 45 ºC, and under 0.3 bar oxygen pressure (Gamelas et al. 2005). This urged the search for alternative efficient methods for oxidation of Mn^{II}-substituted POMs.

In contrast to laccase, versatile peroxidase (VP) produced by fungi of the genera *Pleurotus* and *Bjerkandera* is a high redox-potential enzyme able to oxidize a variety of substrates, including free Mn^{II}, due to the presence of different catalytic sites in its molecular architecture (Ruiz-Dueñas et al. 2009). VP is activated by H_2O_2 in a two-electron reaction yielding highly reactive intermediate states. Activated VP can oxidize two molecules of substrate consecutively in two successive one-electron reactions.

In the present work, reoxidation of $SiW_{11}Mn^{II}$ by the VP/H_2O_2 system was studied for the first time. Based on the easy oxidation of Mn^{II} (as a free ion or in POM complexes) by the enzyme, a novel approach for the delignification catalysis was developed. Reduced POM in the liquor from eucalypt pulp delignification stage was reoxidized by VP, and the resultant liquor mixed with the partially delignified pulp for a further delignification stage in a simple POM-VP-POM trial. In addition, delignification of eucalypt pulp by POM in a VP-assisted process was tested as a pre-bleaching stage to substitute the first ClO_2 stage in a conventional elemental chlorine free (ECF) bleaching sequence.

2. Materials and Methods

The delignification assays were carried out with *Eucalyptus globulus* unbleached kraft pulp supplied by ENCE pulp mill (Spain). The pulp had a kappa number of 13.7, and an intrinsic viscosity of 1180 cm^3/g. For the delignification experiments, a solution containing 2.8 ± 0.1 mmol/L of $[SiW_{11}Mn^{III}(H_2O)O_{39}]^{5-}$ was prepared as previously reported (Galli et al. 2007).

Recombinant VP was obtained from *E. coli* W3110 transformed with the pFLAG-VPL2 expression vector as previously described (Pérez-Boada et al. 2002). Oxidation of $SiW_{11}Mn^{II}$ was followed at 20 ºC in a quartz cell (1 cm optical path) under stirring: 3.0 mL of 0.1 M acetate solution (pH 4.5) containing 2.7 mM $K_6[SiW_{11}Mn^{II}(H_2O)O_{39}].10H_2O$, 0.56-1.26 µM VP and 0.57-2.24 mM H_2O_2 were mixed inside the cell. The increase of absorbance at 490 nm ($SiW_{11}Mn^{III}$ ε_{490} 325 $M^{-1}\,cm^{-1}$; and $SiW_{11}Mn^{II}$ ε_{490} 22 $M^{-1}\,cm^{-1}$) was followed during 1 min intervals, until the reaction reached a plateau. For the assays with the delignification liquor, the H_2O_2 amount varied between 0 and 2.06 mM, with the amount of enzyme kept at 1.20 µM (41.4 mg/L).

Pulp delignification with oxygen was carried out in a PARR reactor, model 4843 (0.25 L) equipped with an automatic temperature control system, pressure control (P_{O2} of 0.5 MPa) and mechanical stirring (220 rpm). Typically 7.5 g of pulp (dry weight), 67 mL of 0.2 M sodium acetate (pH 4.5), 13 mL of 28 mM POM ($SiW_{11}Mn^{III}$) solution, and water to make a final volume of 132 mL were put inside the reactor. The final concentration of POM was 2.7 mM. At the end of the reactions carried out at 110ºC, the reactor was quickly cooled with water and degasified.

In the two-stage experiments, including intermediate POM reoxidation with VP (POM-VP-POM_{reox}), the pulp from the first stage was filtered and pressed, the required amounts of enzyme and H_2O_2 previously optimized to attain near 100% POM reoxidation (POM/VP and H_2O_2/POM molar ratios of 2200 and 0.4, respectively) were added to the delignification liquor, and the solution was stirred at 20-25 ºC for 10 min. The liquor containing the reoxidized POM (verified by visible absorption spectrophotometry) was mixed again with the filtered pulp and a second delignification stage was applied under the same experimental conditions of the first stage. A two-stage experiment not including the reoxidation step of POM by VP/H_2O_2 was also performed by adding fresh POM ($SiW_{11}Mn^{III}$), acetate buffer and water to the washed pulp obtained after the first stage.

Bleaching with ClO_2 was performed on untreated kraft pulp and with pulp delignified with POM, at 10% pulp consistency, in plastic bags in a Grant model Y28 thermostatic bath. The bleaching conditions in the D-Ep-D-Ep-D sequence were as follows: first D stage at 50 ºC for 1 h; second D stage at 70 ºC for 2 h; third D stage at 70 ºC for 2.5 h; first Ep stage at 70 ºC for 1 h, using 2.0% NaOH and 0.2% H_2O_2; second Ep stage at 70 ºC for 1 h, using 1.5% NaOH and 0.1% H_2O_2.The pulp delignified with POM (2 h)-VP-POM_{reox} (2 h) and extracted with NaOH was subjected to D-Ep-D bleaching (POM-VP-POM_{reox}E-D-Ep-D sequence). The conditions of the last stages in this sequence were as follows: first D stage at 50 ºC for 1 h; second D stage at 70 ºC for 2.5 h; Ep stage at 70 ºC for 1 h, using 1.5% NaOH and 0.2% H_2O_2. The loads of ClO_2 for each stage in both sequences are discussed in the text.

The treated pulps were characterized for the kappa number and viscosity according to SCAN methods. Hexenuronic acid content was determined by acid hydrolysis in sodium formate (pH 3.0) followed by spectrophotometric (245 nm) quantification of the furan derivatives formed.

3. Results and discussion

3.1 Kinetics of $SiW_{11}Mn^{II}$ oxidation by VP

The possibility of oxidation of $SiW_{11}Mn^{II}$ employing VP was demonstrated for the first time. Similarly to known oxidation of free Mn^{2+} ions in solution, Mn^{II} in POM was oxidized to Mn^{III} giving rise to the $SiW_{11}Mn^{III}$ species, being active in lignin oxidation. The steady-state kinetic constants for $SiW_{11}Mn^{II}$ oxidation were assessed by non-linear fitting of initial velocities *vs* substrate concentration using the Michaelis-Menten kinetic model (Fig. 1). High VP turnover on $SiW_{11}Mn^{II}$, with a k_{cat} of 47 ± 2 s^{-1}, and a moderate affinity for this compound, with a K_m of 6.4 ± 0.7 mM were revealed. This VP activity was lower than on free Mn^{II}, with a k_{cat} value near 300 s^{-1}, but the main difference between both substrates concerned K_m that was around 0.19 mM for free Mn^{II} ions, revealing over 30-fold higher affinity of VP on the free metal ion. As a result, the global catalytic efficiency of VP oxidizing $SiW_{11}Mn^{II}$ (7.36 ± 0.6 mM^{-1} s^{-1}) was around 200-fold lower than that for oxidation of free Mn^{II} (1600 ± 100 mM^{-1} s^{-1}). This difference may be explained by different accessibility of catalytic sites in VP to $SiW_{11}Mn^{II}$ and free Mn^{II} which needs further investigation.

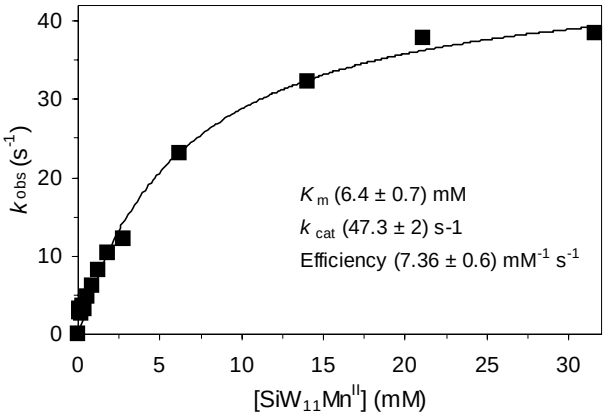

Figure 1. Michaelis-Menten kinetics of $SiW_{11}Mn^{II}$ oxidation by VP.

3.2 Optimization of $SiW_{11}Mn^{II}$ oxidation by VP

A set of assays was carried out to optimize the oxidation of $SiW_{11}Mn^{II}$ to $SiW_{11}Mn^{III}$ by VP in the presence of H_2O_2, either by using an aqueous solution of $SiW_{11}Mn^{II}$ (pH 4.5) at 20 ºC, with 2.7 mM POM concentration, and the liquor from eucalypt kraft pulp delignification obtained with POM (2.7 mM) at 110ºC, pH 4.5, and pulp consistency of ca 5.5%. The assays were performed varying the H_2O_2/POM (0-1.0) and POM/VP (2000-5000) molar ratios. In the experiments with $SiW_{11}Mn^{II}$ solution, the extent of POM oxidation (for a fixed amount of enzyme) increased with the H_2O_2/POM molar ratio until a 0.5-0.6 ratio, and then decreased at higher ratios. Using this H_2O_2/POM ratio (0.5-0.6), 95-100% POM oxidation was obtained in less than 5 min, with a POM/VP ratio ~2200. These values were in agreement with the stoichiometry of the overall enzymatic reaction, which predicts that 0.5 mol of H_2O_2 will be needed to oxidize 1 mol of $SiW_{11}Mn^{II}$. For the H_2O_2/POM ratio of 0.8, only 57% oxidation of $SiW_{11}Mn^{II}$ was obtained, indicating enzyme inactivation by the excess of H_2O_2. If the amount of enzyme was reduced to about 50%, keeping the H_2O_2/POM ratio of 0.5, the oxidation extent also decreased (to only 55%) due to the increased H_2O_2/VP ratio. However, when the later assay was carried out by adding the H_2O_2 in several steps, without exceeding a 500-fold molar excess of H_2O_2 in each addition, the extent of oxidation (94%) was similar to that attained using a higher amount of enzyme. These data confirmed VP inactivation by H_2O_2 (even in the presence of enough amount of $SiW_{11}Mn^{II}$ to

consume all the H_2O_2) and showed that the enzyme dose can be reduced by stepwise addition of H_2O_2 (to prevent VP inactivation). The POM oxidation was not observed only with H_2O_2 nor only with the enzyme. The highest oxidation degree (over 90%) of POM in the liquor from delignification of eucalypt kraft pulp was obtained at H_2O_2/POM/VP ratios similar to those mentioned above. Hence it was concluded that a H_2O_2/POM ratio around 0.5 and a POM/VP ratio of 2000-3000 should be used to obtain near complete reoxidation of the manganese-substituted POM.

3.3 VP-assisted two-stage pulp delignification by POM

Following the promising results on $SiW_{11}Mn^{II}$ reoxidation by VP, short delignification sequences with POM have been carried out using intermediate step with VP. Hence first delignification stage with $SiW_{11}Mn^{III}$ and O_2 (POM stage) was followed by pulp filtration, and a short intermediate step has been applied consisting of the addition of VP and H_2O_2 to the filtrate. The reoxidised $SiW_{11}Mn^{II}$ solution was used again in the POM stage. The results were compared with those obtained when the second delignification stage was performed by adding chemically-prepared $SiW_{11}Mn^{III}$, as well as when only one-stage POM delignification was performed (Table I).

Table I. Delignification of eucalypt kraft pulp with $SiW_{11}Mn^{III}/O_2$ assisted by VP/H_2O_2[a]

	Kappa number	Viscosity (cm^3/g)	Kappa decrease (%)[d]	Viscosity loss (%)	HexA (mmol/kg)
Initial kraft pulp	13.6	1215	-	-	61.2
O_2 (without POM, 2 h)	7.3	875	46 (33)	28	15.7
POM (1 h)	8.2	1180	40 (33)	3	28.5
POM (2 h)	6.8	1140	50 (40)	6	18.4
POM (1 h)-VP-POM (1 h)[b]	6.5	1130	52 (42)	7	16.8
POM (2 h)-VP-POM (2 h)[b]	5.2	1080	62 (51)	11	9.3
POM (2 h)-POM (2 h)[c]	5.2	1085	62 (50)	11	8.2

[a] Pulp consistency of 5.4%; 2.7 mM POM; pH 4.5; pO_2 of 0.5 MPa; 110 ºC; and 220 rpm
[b] The pulp after the first stage was filtered, and the POM in the filtrate reoxidized by VP/H_2O_2.
[c] The pulp after the first stage was washed, and fresh POM ($SiW_{11}Mn^{III}$) was added
[d] In parentheses is a Kappa number reduction corrected for HexA ($kappa_{cor}$= kappa - 0.073 × [HexA])

After one-stage POM delignification at 110 ºC, decreases of kappa number (a rough measure of the lignin content in pulp) of 40% and 50%, with viscosity losses of only 3% and 6%, were obtained after 1-h and 2-h reaction, respectively (Table I). Besides residual lignin, hexenuronic acids contribute significantly to the kappa number in *E. globulus* kraft pulps and to the consumption of bleaching reagents. In fact, a significant removal of hexenuronic acids (up to 70% after 2 h) was detected after the POM treatment. It is noteworthy that the POM/O_2 system was highly selective for delignification when compared with the oxygen-delignification control, which showed a viscosity loss of 28% (near 5-fold higher than that obtained with POM delignification) (Gaspar et al. 2003).

After two-stage (2-h each) POM delignification including the intermediate reoxidation step with VP and H_2O_2, kappa number was reduced by 62% and the viscosity dropped by 11% (Table I). Interestingly, this treatment also degraded almost 90% of the hexenuronic acids present in the pulp. The delignification degree corrected for the hexenuronic acids content was 51%. Similar

results in terms of pulp kappa number, viscosity and hexenuronic acid degradation were obtained in parallel assays with freshly-prepared $SiW_{11}Mn^{III}$ added after the first POM stage, revealing that the presence of the enzyme did not exert a negative effect on the performance and selectivity of the $SiW_{11}Mn^{III}/O_2$ system.

3.4 ECF bleaching with a VP-assisted pre-bleaching POM stage

Pulp treatment with the VP-assisted two-stage POM delignification (2-h each followed by an alkaline extraction (POM-VP-POM_{reox}-E) was investigated to substitute the first ClO_2 stage in a conventional D-Ep-D-Ep-D ECF bleaching sequence for eucalypt kraft pulp. Results from the conventional D-Ep-D-Ep-D bleaching sequence (see Materials and Methods section) and the sequence including VP-assisted two-stage POM delignification, (POM-VP-POM_{reox}E-D-Ep-D), were compared in terms of ClO_2 savings for the same final brightness (~89% ISO). Pulp bleached by the sequence including VP and POM showed a ClO_2 consumption 50% lower than the conventional ECF sequence (Table II). The ClO_2 oxidation equivalents (OXE) per kappa number unit in the modified sequence were higher than in the conventional sequence indicating the worst oxidability of residual lignin by ClO_2 after POM stages. Furthermore, the main strength properties of the unbeaten pulps after the two bleaching sequences were similar (results are omitted). The results obtained suggest that VP-assisted continuous reutilization of $SiW_{11}Mn^{III}$ in a two-reactor system may be implemented in future industrial ECF sequences, with no apparent deterioration of the pulp strength properties, while significantly reducing the ClO_2 consumption, and consequently lowering the environmental impact of the bleaching process.

Table II. ClO_2 consumption in D stages of conventional ECF sequence and with POM-VP-POM pre-bleaching stage (pulp brightness of 89% ISO)

	D-Ep-D-Ep-D	POM-VP-POM_{reox}E-D-Ep-D
ClO_2 consumption [a]	25 + 9 + 6	15 + 5
OXE [b]	90	134

[a] As active chlorine in each D stage (kg/ton)
[b] As moles of active chlorine per ton of dry pulp and per kappa unit

4. Conclusions

The possibility of oxidation of Mn-substituted POM, $SiW_{11}Mn^{II}$, into the analogue $SiW_{11}Mn^{III}$, by VP in the presence of H_2O_2 was demonstrated for the first time. This POM, whose oxidized form is highly selective in delignification of kraft pulp, was fully oxidized by VP/H_2O_2 at 20-25 ºC in less than 10 min. In this way, a continuous POM-catalyzed delignification process is possible while applying an intermediate step consisting of the re-oxidation of reduced POM by VP/H_2O_2. The highly selective kraft pulp delignification was demonstrated in a short trial POM-VP-POM resulting in 62% reduction of the pulp kappa number and a viscosity loss of only 10%. The substitution of the first ClO_2 stage by a POM-VP-POM_{reox} treatment in a conventional DEDED bleaching sequence allowed 50% ClO_2 savings for the same final pulp brightness without decreasing of pulp strength properties.

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